IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Takeshi KOIZUMI, et al.

Serial No.: 10/601,713 Group Art Unit: 1634

Filed: June 20, 2003 Examiner: Bausch, Sarael

For: Process for Improving Efficiency of DNA Amplification Reaction

DECLARATION UNDER 37 CFR §1.132

I, Hitoshi AOKI, citizen of Japan, residing at 27-18-303, Makuhari-Hongo 7, Hanamigawa-ku, Chiba City, Chiba, Japan, hereby declare that:

1. I received the Ph. D. degree in the Science of Agriculture from The University of Tokyo, Tokyo, Japan in 2004.

2. I am currently employed by Nichirei Foods Inc. and began working for Nichirei Corporation (a holding company of Nichirei Groups including Nichirei Foods Inc.), in April 1995, where I have engaged in research and development relating to functionality of food materials. I have a through knowledge of Biology and Genetic Engineering, and the recent academic achievements include the following publications:

1) Aoki, H., Ahsan, M. N., Matsuo, K., Hagiwara, T., Watabe, S. (2003)
Purification and characterization of collagenolytic proteases from the hepatopancreas of northern shrimp (Pandalus eous).
Journal of Agricultural and Food Chemistry 51, 777-783.

2) Aoki, H., Ahsan, M. N., Watabe, S. (2003)
Molecular cloning and characterization of cathepsin B from the hepatopancreas of northern shrimp Pandalus borealis.
Comparative Biochemistry and Physiology 134B, 681-694.

3) Aoki, H., Ahsan, M. N., Watabe, S. (2003)
Molecular cloning and functional characterization of crustapain: a distinct cysteine proteinase with unique substrate specificity from northern shrimp Pandalus borealis.
The Journal of Biochemistry 133, 799-810.

4) Aoki, H., Ahsan, M. N., Watabe, S. (2003)
Heterologous expression in Pichia pastoris and single step purification of a cysteine proteinase from northern shrimp.
Protein Expression and Purification 31, 213-221.

5) Aoki, H., Ahsan, M. N., Watabe, S. (2004)
Molecular and enzymatic properties of a cathepsin L-like proteinase from northern shrimp (Pandalus borealis) with distinct substrate specificity.
Journal of Comparative Physiology B 174, 59-69.

6) Aoki, H., Ahsan, M. N., Matsuo, K., Hagiwara, T., Watabe, S. (2004)
Partial purification of proteases that are generated by processing of the Northern shrimp Pandalus borealis and which can tenderize beef.
International Journal of Food Science and Technology 39, 471-480.

7) Ahsan, M. N., Aoki, H., Watabe, S. (2005)
Overexpression in E. coli and functional reconstitution of anchovy trypsinogen from inclusion body.
Molecular Biotechnology 30, 193-205.

3. I have read Application No. 10/601,713 of Takeshi KOIZUMI et al., filed June 20, 2003, and entitled "Process for Improving Efficiency of DNA Amplification

Reaction". I have also read the Advisory Action dated March 16, 2006 and the final Office Action dated November 30, 2005 and the references cited therein, including Sorensen et al. J. Virology, Dec. 1993, p. 7118-7124, in connection with Application No. 10/601,713.

4. Sorensen et al., hereinafter referred to as "the citation", discloses a method of amplifying DNA by PCR. The citation describes, on the left column of page 7120, that oligonucleotides (primers) were prepared using an automated DNA synthesizer manufactured by Applied Biosystems. Attached is an excerpt of a user's manual that can be downloaded from the following site.
http://dosc.appliedbiosystems.com/search.taf
I submit the excerpt as an exhibit to support my declaration.

5. The excerpt corresponds to the section 2 of the manual and is related to the chemistry of automated DNA synthesis. The last paragraph on page 2-31 of this manual reads "The deprotected, detritylated DNA has a free 5' and 3' hydroxyl and is biologically active." Figure 2-8 on page 2-32 shows the DNA has 5' hydroxyl.

6. I have examined the content of the citation and have found nothing indicating or suggesting that the method

of Sorensen utilized a primer with a phosphate group at the 5' terminus. Accordingly, there is no doubt that the primer used in the citation has a hydroxyl group at the 5' terminus.

7. I have also examined the citation for the description regarding the degeneracy of the biotinylated primer. The citation discloses that biotinylated primer is not degenerated as shown in Fig. 1 of the citation. The flanking primer (FP), that is not biotinylated and used in combination with the biotinylated primer, has a degenerate sequence as shown in panel C of Fig. 1. None of the primers 1 to 4 has a degenerate sequence as shown in the panel C. Therefore, I believe that the citation neither teaches nor suggests use of a set of biotinylated non-degenerate primers.

8. I have also examiner the citation for the type of PCR and found no description indicating that the method of Sorensen may be performed with asymmetric PCR.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such

willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: May 22, 2006

By: Hitoshi Aoki

Hitoshi AOKI

Exhibit

Model 380B DNA Synthesizer

Version 1.1

User's Manual

Applied Biosystems

SECTION 2

CHEMISTRY FOR AUTOMATED DNA SYNTHESIS

Contents:

INTRODUCTION

DNA synthesis is quite simple in concept. A reactive 3' phosphorous group of one nucleoside is coupled to the 5' hydroxyl of another nucleoside. The former is a monomer, delivered in solution. The latter is immobilized on a solid support. An internucleotide linkage is thus formed. Three other chemical reactions are necessary to prepare the growing chain of DNA for the next coupling. In this way a synthesis cycle is conducted, adding one nucleoside monomer at a time. The desired sequence and length are defined by the operator on the synthesizer[1]. When the chain is complete, the crude DNA (*oligonucleotide*) must be *cleaved* from the support and *deprotected*. Further purification is usually advised. When the complete operation becomes routine, the synthesis of oligonucleotides becomes reliable and their biological activity is assured. Since most laboratories are not interested in this as a research project, the goals are for DNA synthesis to become cheaper, faster, better, easier, and more flexible. This chapter will help you understand the synthesis chemistry and how you can attain these goals.

The *phosphoramidite* method of oligonucleotide synthesis is the chemistry of choice for most laboratories because of efficient and rapid coupling and the stability of the starting materials[2]. The synthesis is performed with the growing DNA chain attached to a solid support so that excess reagents which are in the liquid phase can be removed by filtration[3]. Therefore, no purification steps are required between cycles. This support material is a form of silica, controlled-pore-glass (*CPG*) beads[4]. The particle size and the pore size have been

optimized for liquid transfer and mechanical strength. The synthesis cycle is depicted in Figure 2-1. The starting material is the solid support derivatized with the nucleoside which will become the 3'-hydroxyl end of the oligonucleotide. As shown in Figure 2-2, the nucleoside is bound to the solid support through a *linker* attached at the 3'-hydroxyl. The 5'-hydroxyl is blocked with a *dimethoxytrityl* (DMT) group.

The first step of the synthesis cycle is treatment of the derivatized solid support with acid to remove the DMT group (Figure 2-4). This frees the 5'-hydroxyl for the coupling reaction (Figure 2-5). An activated intermediate is created by simultaneously adding the phosphoramidite nucleoside monomer and tetrazole, a weak acid, to the reaction column. The tetrazole protonates the nitrogen of the phosphoramidite, making it susceptible to nucleophilic attack. This intermediate is so reactive that addition is complete within 30 seconds. As shown in Figure 2-5, the phosphoramidite is blocked at the 5'-OH with the dimethoxytrityl group.

The next step, capping, terminates any chains which did not undergo addition. Since the unreacted chains have a free 5'-OH, they can be terminated or capped by acetylation. These unreacted chains are also called "failure products". Capping is done with acetic anhydride and 1-methylimidazole[5]. Since the chains which reacted with the phosphoramidite in the previous step are still blocked with the dimethoxytrityl group, they are not affected by this step. Although capping is not required for DNA synthesis, it is highly recommended because it minimizes the length of the impurities and thus

facilitates product identification and purification (Figure 2-6).

The internucleotide linkage is then converted from the phosphite to the more stable phosphotriester. Iodine is used as the oxidizing agent and water as the oxygen donor. This reaction is complete in less than 30 seconds (Figure 2-7).

After oxidation, the dimethoxytrityl group is removed with a protic acid, either trichloroacetic or dichloroacetic acid. The cycle is repeated until chain elongation is complete. At this point, the oligonucleotide is still bound to the support with protecting groups on the phosphates and the exocyclic amines of the bases A, G, and C. The oligonucleotide is cleaved from the support by a one-hour treatment with concentrated ammonium hydroxide. Ammonia treatment also removes the cyanoethyl phosphate protecting groups. The crude DNA solution in ammonium hydroxide is then treated at 55°C for 8 to 15 hours to remove the protecting groups on the exocyclic amines of the bases (Figure 2-8).

Note that synthesis can be performed using methyl, or the newer cyanoethyl phosphoramidites[6]. These two versions of synthesis monomers differ only by the protecting group on the phosphorous oxygen. The synthesis chemistry and the resulting oligonucleotide are the same with both. Excellent results are obtained with either one[7]. The primary difference is that when using methyl phosphoramidites, thiophenol treatment is required to deprotect the internucleotide methyl phosphotriester groups at the end of synthesis. Thiophenol is foul smelling and toxic. In addition, this adds an extra 30-60 minutes

to deprotection time. For these reasons, use of cyanoethyl phosphoramidites is strongly recommended. Methyl phosphoramidites, since they offer no advantage or unique utility, are no longer available from Applied Biosystems.

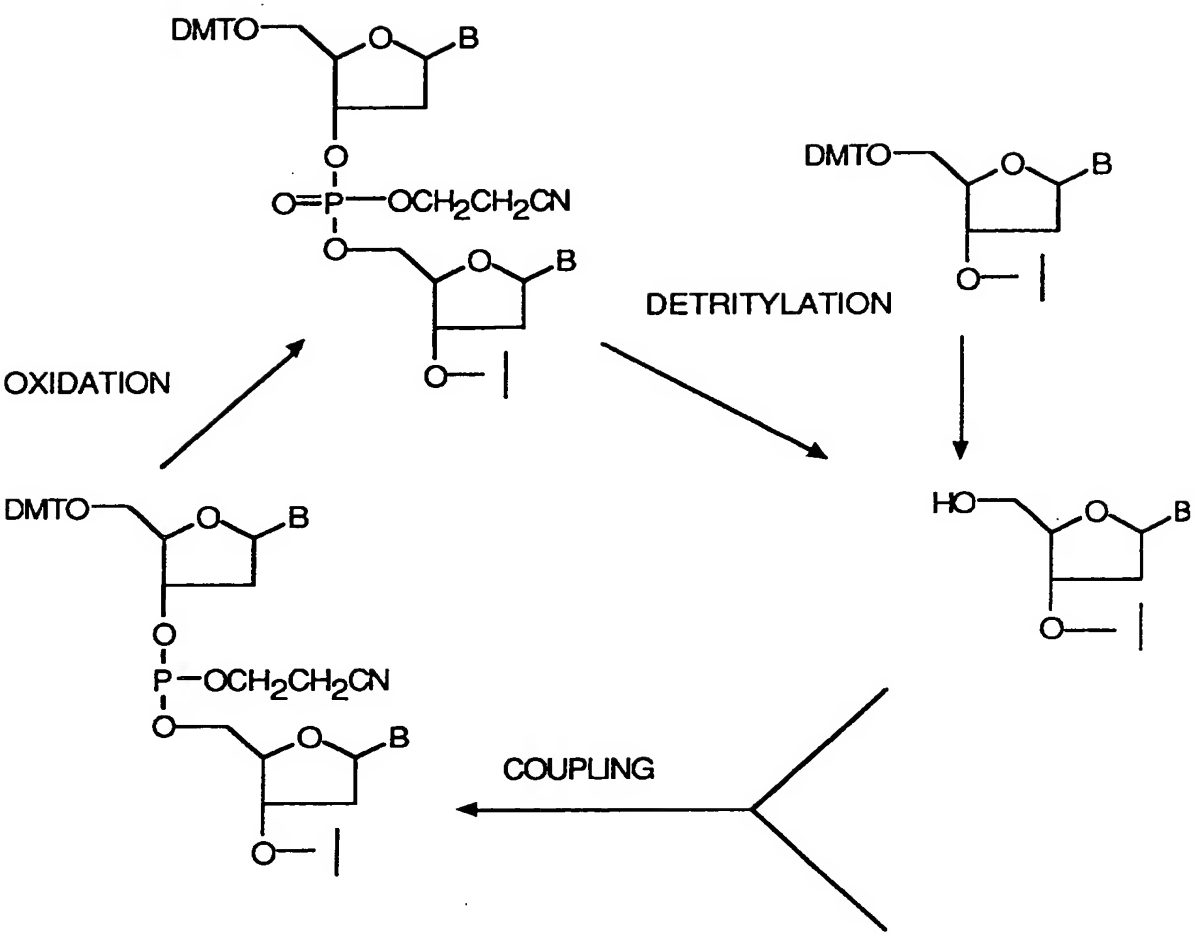


Figure 2-1
The Synthesis Cycle

THE SOLID SUPPORT - CPG

The Model 380B DNA Synthesizer uses a solid phase synthesis chemistry in which the growing DNA chain remains covalently attached to an insoluble support. All reagents and solvents flow through the support which is contained within a synthesis column.

The support used for DNA synthesis is Controlled-Pore-Glass (CPG)[4]. When used with the Model 380B and other Applied Biosystems reagents, CPG produces coupling efficiencies of 98 to 100%, as measured by the trityl cation assay. This enables the synthesis of oligomers up to 175 bases in length[8]. CPG is a porous, non-swelling particle which is about 150 microns in diameter and has 500Å pores. A wide-pore CPG support (1000Å) is also available and should be used when synthesizing oligonucleotides greater than 70 bases[8]. CPG is covalently derivatized with one of the four nucleosides (A, G, C or T), see Figure 2-2. The reactive groups on these nucleosides are blocked or protected to prevent unwanted side reactions. They are all blocked at the 5'-hydroxyl with a dimethoxytrityl group. As shown in Figure 2-3, the exocyclic amines on adenosine (A) and cytosine (C) are protected by a benzoyl group (A^{bz}, C^{bz}), and the exocyclic amine on guanosine (G) by an isobutyryl group (G^{ib}). Thymidine does not need a protecting group since the thymine ring has no exocyclic amines.

As shown in Figure 2-2, CPG has a linker attached to its surface via a siloxane bond. All free silanol groups are capped to prevent side reactions. The support is then derivatized by covalently attaching the 3'-hydroxyl of the nucleoside to the linker via a

succinate ester bond. The bond is base labile and allows for removal of the DNA from the support with ammonia. After synthesis is complete the oligonucleotide is quantitatively cleaved with a free 3'-hydroxyl.

The loading of nucleoside as measured by DMT release is 27 to 30 micromoles per gram of 500Å support. Prefilled columns contain 0.2 µmol, 1 µmol or 10 µmol of initial nucleoside. The 0.2 µmol scale provides sufficient amounts of purified oligomers for most applications. The 1 µmol scale is used when greater quantities of DNA are needed. The 10 µmol scale is useful for physical studies, such as X-ray crystallography or NMR. Wide-pore CPG supports are only available on the 0.2-micromole level. The loading of nucleoside is lower, about 15 µmol/gm of 1000Å support. For the synthesis of long oligonucleotides ("bigmers"), it has been shown that a lower loading of nucleoside and larger CPG pore size are critical requirements for success.

Typical quantities of oligonucleotide obtained from the different synthesis scales are given below:

synthesis cycle	crude yield*(O.D.) (20mer)
0.2μM	20-25
1 μM	100-120
10 μM	800-1000

* Yield figures based on a 20mer sequence. Absorbance measured at 260nm, assuming 33 micrograms/O.D. unit.

With automated synthesis, the DNA is built from 3' to 5'. Before beginning a synthesis, one of four support-bound nucleosides (A, C, G or T) contained within a column is placed on the instrument. This nucleoside is the 3'-terminus of the sequence.

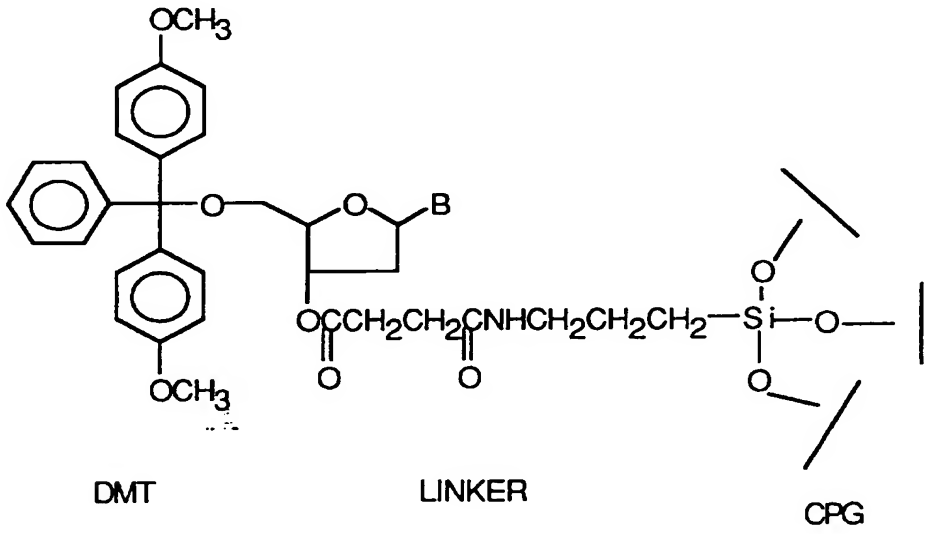
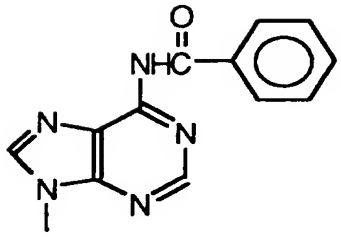
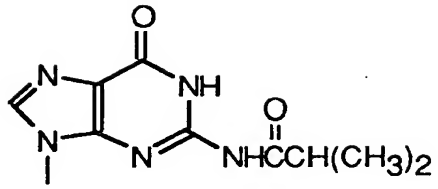


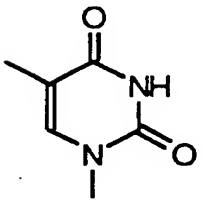
Figure 2-2
The DMT protected nucleoside is attached to the controlled pore glass (CPG) support
B = Base, A,G,C,T).



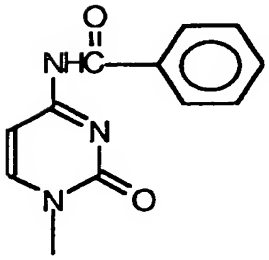
Adenosine-benzoyl protected



Guanosine-isobutyryl protected



Thymidine



Cytosine - benzoyl protected

Figure 2-3
Protected exocyclic base amines. Adenosine (A) and cytosine (C) are protected by a benzoyl group (Bz), and guanosine (G) by an isobutyryl group (Ib). Thymidine (T) does not need a protecting group.

DNA SYNTHESIS CHEMISTRY CYCLE

Each cycle of base addition consists of four steps:

1) Detritylation
2) Coupling
3) Capping
4) Oxidation.

These reaction steps are repeated in the above order until all bases are added. Following synthesis, the DNA chain is cleaved from the solid support and the cyanoethyl phosphate protecting groups are removed automatically. The Model 380B does not base deprotect the DNA. That must be completed manually. Each step of the synthesis cycle will be discussed in detail.

Detritylation

The first step in oligonucleotide synthesis is to remove the acid-labile, dimethoxytrityl (DMT) protecting group on the 5'-hydroxyl of the support-bound nucleoside. As shown in Figure 2-4, treatment with the protic acids, trichloroacetic acid (TCA) or dichloroacetic acid (DCA), will deprotect or detritylate the 5'end. This will yield a reactive 5' hydroxyl which can couple with a phosphoramidite during the following addition step. The two acids can be used interchangeably and are both available from Applied Biosystems. Dichloroacetic acid is a weaker acid and may yield lower levels of depurination in the synthesis of long oligonucleotides. However, this observation has not been consistently corroborated. For convenience, in this manual the detritylation acid will exclusively be referred to as TCA.

Detritylation in more detail:

Immediately before detritylation, the CPG support is washed with acetonitrile to eliminate traces of the preceding reagent. Next, TCA (bottle 14) is delivered to the column to cleave the trityl group. Detritylation under anhydrous conditions, is a reversible reaction. The DMT cation is highly reactive and can re-tritylate any reactive nucleophile. Detritylation is driven to completion by the removal of the DMT cation from the synthesis column. Therefore, detritylation is conducted by continuous delivery of TCA and elution of the DMT cation. Unlike the other reactions in the cycle, there is no wait period for detritylation. Any residual TCA must be removed by an acetonitrile wash to prevent detritylation of the incoming phosphoramidite. If the phosphoramidite monomer becomes detritylated, an unwanted dimer can form in solution and then couple to the support-bound nucleotide chain. Continued chain propagation would result in some sequences being longer than the product, making purification difficult.

Following both acetonitrile washes, the remaining solvent is forced out of the column by an *argon reverse flush* - argon passes through the column from top to bottom and pushes the liquid to waste. For a summary of these steps, see Table 2-1.

TABLE 2-1

DETRITYLATION STEPS

STEP DESCRIPTION	PURPOSE
acetonitrile (bottle 18) delivery to column	wash column and support, remove traces of preceding reagent
argon reverse flush	remove residual acetonitrile from column
TCA (bottle 14) delivery to column	detritylate support-bound nucleoside
acetonitrile (bottle 18) delivery to column	wash column, remove traces of TCA
argon reverse flush	remove residual acetonitrile from column

NOTE: The complete synthesis cycle contains steps to wash and flush the valve blocks and delivery lines.

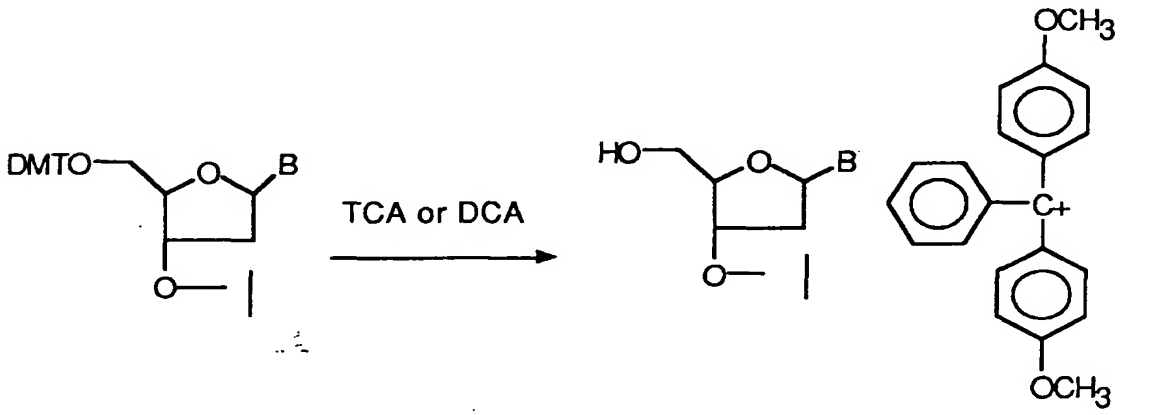


Figure 2-4

Detritylation. TCA or DCA is used to remove the DMT from the 5'end. This leaves a 5' hydroxyl to react with the incoming phosphoramidite in the coupling step. Quantitating the released trityl cation indicates the step-wise yield and can be used to monitor the instrument's performance.

Monitoring the Trityl Cation:

When the dimethoxytrityl, also referred to as DMT or trityl, protecting group is cleaved from the nucleotide, it exists as a cation (Figure 2-4). When in acid solution, this cation is relatively stable and produces a brilliant orange color. It has an absorbance maximum at about 498 nm and extinction coefficient of about 70,000 in most solvents, such as acetonitrile or dichloromethane. It is easily detected and quantitated spectrophotometrically.

To quantitate the trityl cation released at each detritylation step, the column effluent from the TCA delivery and the subsequent wash with acetonitrile is collected. Instead of the effluent flowing into the waste bottle, it is channelled through the *trityl collection port* into tubes in a fraction collector. The Model 380B cycles provide for the collection of trityl effluent fractions and include a signal to advance a fraction collector (user provided).

Next, the absorbance of the fractions is measured to quantify the trityl released in each addition cycle. Since the trityl solution is very concentrated, it must be diluted before quantitating or significant errors in the readings will occur. Typically, the trityl fraction is brought up to a volume of 10 mL with 0.1 M p-toluenesulfonic acid in acetonitrile. The trityl yield is then used to calculate the coupling efficiency of each addition step by dividing the next step absorbance value from the previous step value. From this data, an overall stepwise yield can be determined and the expected product yield can be estimated. The molar amount of DMT cation can be calculated using Beer's Law:

$A = eCl$ where: A - absorbance
e - extinction coefficient
C - concentration
l - path length

$$\text{overall yield} = (\text{coupling yield})^{\text{number of couplings}}$$

For more details, see the trityl assay procedure discussed in User Bulletin 13 (Revised) in the appendix.

Monitoring the trityl cation is very important, but the results must be interpreted with caution. The trityl assay is only an indirect measure of synthesis efficiency. Certainly, high trityl cation yields (>98%) must be present for good synthesis. However, high trityl yields can be present when a poor synthesis occurs. The reason for this discrepancy is that although this chemistry is highly refined, it is not perfect. Certain reactions contribute to the trityl cation released each cycle. In particular, a low level of extraneous chain growth other than the desired oligonucleotide can occur.

One other cause of imperfect results might be incomplete detritylation or capping. This could happen if the synthesizer is not operating correctly, the cycle is not optimized, or the reagents are wet or impure. These occasions generate species other than the desired product which release the trityl cation. Low trityl cation yields (<98%) always predict a less than optimal synthesis. In practical terms, trityl yields of less than 95% will not allow identification or purification of even short oligonucleotides. For the above reasons the trityl assay must be viewed only as a preliminary, yet convenient, monitor of the

synthesizer's performance. The assay is useful to aid in the early detection and diagnosis of instrument related problems. Many laboratories monitor their trityl fractions only by visual inspection. With experience, a failed synthesis is detected this way. Evaluation of the oligonucleotide by PAGE or HPLC is much more informative than the trityl assay. Synthesizer or reagent problems can be adequately diagnosed only by these methods, which are direct analyses of the product.

Depurination:

Trichloroacetic acid is a very effective protic acid detritylating agent. However, in the presence of protic acids, amine-protected purines (A^{bz} and G^{ib}) are susceptible to depurination (removal of the purine from its sugar). The chemical mechanism is initial protonation at N-7 of the purine ring, causing increased lability of the ribose 1' - purine N-9 bond. Cleavage of adenine and guanine bases yields a 1' hemiacetal ribose ring, the result of depurination. Oligonucleotides which contain apurinic sites are cleaved during the ammonium hydroxide treatment. Cleavage occurs at the internucleotide bonds on the 3' hydroxyl side of the apurinic deoxyribose (This is similar in effect to the chemistry of Maxam-Gilbert sequencing). Each purine in the oligonucleotide chain is exposed to acid at each detritylation step. Purines near the 3' end will have the longest cumulative exposure time and a greater chance for depurination. It has been reported that the 5' terminal purine is more susceptible to depurination than an internucleotide purine with a 3' and a 5' phosphorous[9].

The quantity of DNA fragments generated by apurinic ammoniolysis is usually insignificant. If the synthesis is conducted Trityl On, for purification by OPC or Trityl On HPLC, the 5' end fragment of apurinic ammoniolysis will bear a DMT group and may complicate purification. In practice however, using the ABI reagents and cycles optimized for DNA synthesis, depurination is not detectable or significant except for long oligonucleotides (>80 bases) which are purine rich near the 3' end[10]. To minimize depurination, each treatment with TCA should not be extended beyond the times specified in the cycles.

IMPORTANT: Do not stop a synthesis while the DNA is exposed to TCA. Do not increase TCA delivery times.

COUPLING

Phosphoramidites:

Phosphoramidites (shown in Figure 2-5a) are chemically modified nucleosides which are used as the building blocks for synthesizing DNA. They are added to the support-bound nucleotide chain one at a time until all bases in the sequence are coupled. The cyanoethyl phosphoramidite nucleosides have the following functional groups:

1. A diisopropylamino on a 3' trivalent phosphorous moiety[11]. The phosphoramidite is very stable and is made highly reactive by the activator, tetrazole.

2. A ß-cyanoethyl protecting group on the 3' phosphorous moiety. This group prevents side reactions and aids in solubility of phosphoramidites. It is removed upon completion of the synthesis by using ammonia. In deprotection, ammonia acts as a base to remove a proton on the methylene group bearing the nitrile group. This anion is formed only in low concentration, but rapidly fragments by a ß-elimination reaction to form acrylonitrile and the deprotected internucleotide phosphodiester group. Acrylonitrile then reacts irreversibly with ammonia to form 3-aminopropionitrile, an inert compound.

3. A dimethoxytrityl (DMT) protecting group on the 5' hydroxyl. The DMT is removed during each detritylation step leaving a reactive 5' hydroxyl available for coupling an incoming phosphoramidite.

4. A benzoyl protecting group on the exocyclic amines of A and C (A^{bz}, C^{bz}), and an isobutyryl protecting group on the exocyclic amine of G (G^{ib}). These amide groups prevent side reactions and are removed upon completion of the synthesis with ammonia. Since thymidine is unreactive and does not contain an exocyclic amine moiety, it is not protected.

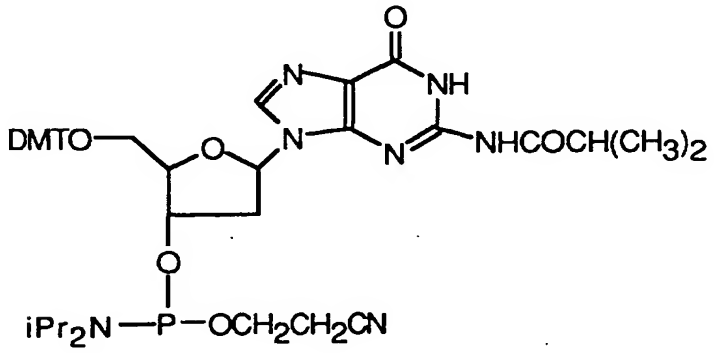


Figure 2-5a
Structure of guanosine cyanoethyl phosphoramidite

Coupling:

Before beginning the coupling step, the support is made anhydrous and free of nucleophiles (e.g. water) by an extensive wash with acetonitrile. Any extraneous nucleophiles will compete with the support-bound 5' hydroxyls for the activated phosphoramidite and will decrease coupling efficiency. The column is then dried by an argon reverse flush to remove residual acetonitrile. Tetrazole, the phosphoramidite activator, is next delivered to the column. According to the oligonucleotide sequence, one or more of the phosphoramidites (bottles 1 to 7) and tetrazole

(bottle 9) are then simultaneously delivered to the column. Depending on the synthesis cycle, alternate deliveries of tetrazole and then base-plus-tetrazole are repeated up to 3 times. When these reagents mix, the mild acid, tetrazole, (pKa = 4.8, Ref.8) transfers a proton to the nitrogen of the diisopropyl group on the 3' phosphorous (See Figure 2-5b). This protonated amine makes a very good leaving group upon nucleophilic attack by the tetrazole to form a tetrazolyl phosphoramidite[12] . This is the reactive intermediate which forms the internucleotide phosphite with the support bound 5' hydroxyl. A molar excess of tetrazole ensures that the phosphoramidite will be activated. The excess of phosphoramidite relative to free 5' hydroxyl ensures that the reaction is nearly quantitative (over 98% coupling). The coupling steps are summarized in Table 2-2.

Mixed sequence probes are synthesized by simultaneous delivery of up to 4 bases and tetrazole with near equivalent coupling. The four nucleoside phosphoramidites have slightly different reactivities, as all different molecules must[13]. The cyanoethyl phosphoramidites follow the reactivity order of T > G > C > A. When all four are delivered simultaneously, their representation will be (normalized to 100%):

T - 30%
G - 26%
C - 24%
A - 20%

These values are slightly dependent on cycle, location of the site in the oligonucleotide, age of the phosphoramidite solutions, etc. They have a range of about 3% because of these variables.

TABLE 2-2

COUPLING STEPS

STEP DESCRIPTION	PURPOSE
acetonitrile (bottle 18) delivery to column	remove nucleophiles render support
argon reverse flush	remove residual
tetrazole (bottle 9) delivery to column	deliver activator
tetrazole + phosphor-amidite delivery to column	activate phosphor-amidite, begin coupling reaction
tetrazole delivery to column	continue coupling
tetrazole + phosphor-amidite delivery to column	continue coupling
wait	complete coupling
argon reverse flush	remove the tetrazole

IMPORTANT: These deliveries are critical. Under-delivery causes low coupling efficiency. Over-delivery wastes reagents.

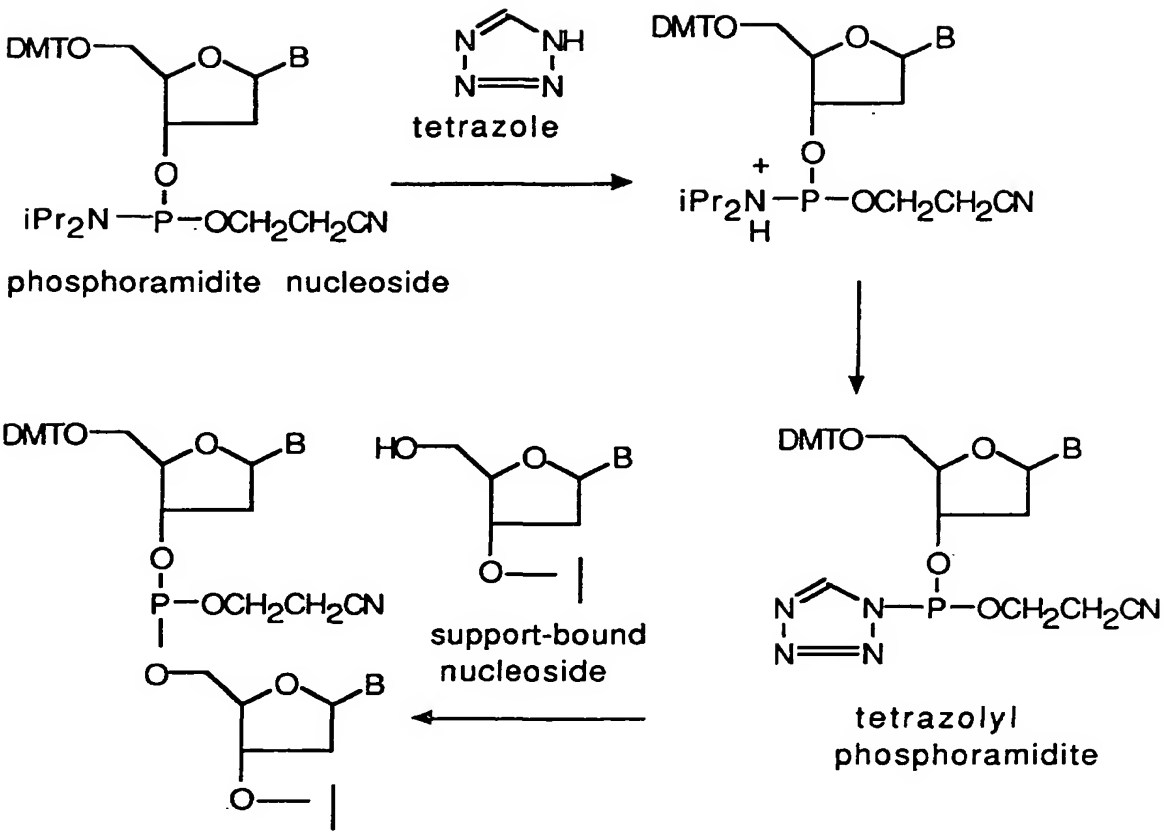


Figure 2-5b
The coupling step. Phosphoramidites and tetrazole are delivered to the column which contains the support-bound nucleotide. The diisopropylamine is protonated and displaced by tetrazole. When the 5' OH couples to the phosphorous, a 5' to 3' internucleotide linkage is created.

CAPPING

Because coupling is not always quantitative, a small percentage (up to 2%) of support-bound nucleotides can fail to undergo addition. These truncated, or *failure sequences*, will remain attached to the support. If they remain in the hydroxyl form, they can propagate in subsequent coupling steps. Failure sequences with one less base than the product would then be generated making isolation of the product more difficult. Capping the remaining free hydroxyls by acetylation eliminates this problem. The capped failure sequences are then prevented from participating in the rest of the synthesis reactions.

To cap, equal volumes and equimolar amounts of two binary reagents, acetic anhydride (bottle 11) and 1-methylimidazole[5], NMI, (bottle 12), are simultaneously delivered to the column. As shown in Figure 2-6, the reagents mix and create a powerful acetylating agent. The two reagents need to be segregated since the active acetylating agent is unstable. This agent reacts at the 5' hydroxyls rendering these moieties unreactive for the remainder of the synthesis. The excess reagents are then removed by an argon reverse flush. The capping time required to acetylate the 1 or 2% unreacted 5' hydroxyls is very brief, only a few seconds. It is important to minimize this time to prevent loss of cyanoethyl groups from the internucleotide linkages and to prevent base modification by-products. The efficiency of shorter capping time and the capping/oxidation order have been extensively demonstrated in studies at Applied Biosystems[14].

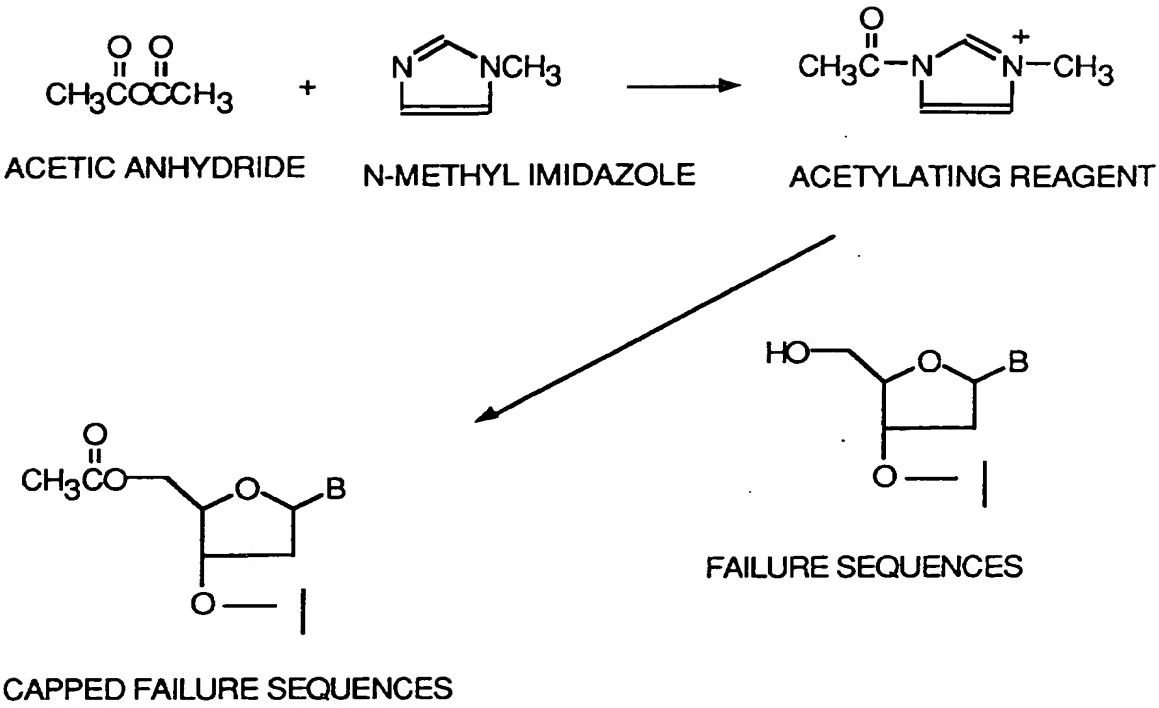


Figure 2-6
Capping of unreacted chains. The capping reagents, acetic anhydride and 1-methylimidazole (NMI), are used to terminate unreacted chains by acetylating the 5' hydroxyl groups.

OXIDATION

The newly formed internucleotide linkage is a phosphite (trivalent phosphorous) triester. The phosphite linkage is unstable and is susceptible to acid and base cleavage. Therefore, immediately after capping, the trivalent phosphite triester is oxidized to a stable pentavalent phosphate triester. This is shown in Figure 2-7.

Oxidation follows capping to eliminate the possibility of traces of water from the oxidizing solution causing acetic anhydride to form acetic acid during capping. This would expose the oligonucleotides to acid as well as make capping less effective. Also, scientists at Applied Biosystems have elucidated the complex chemical pathway whereby a small side-reaction, the phosphitylation of the O-6 position of guanosine can be minimized when capping immediately follows coupling[5,15]. The enzymatic digestion/base composition assay on oligonucleotides made with different cycle orders, capping then oxidation and oxidation then capping, shows markedly different results. The Applied Biosystems standard, capping then oxidation, virtually eliminates base-modified nucleosides.

Iodine is used as a mild oxidant in a basic tetrahydrofuran (THF) solution with water as the oxygen donor. When the iodine-water-pyridine-THF mixture (bottle 15) is delivered to the column, an iodine-pyridine complex forms an adduct with the trivalent phosphorous. This adduct is decomposed by water with production of a pentavalent phosphotriester internucleotide group. This is an extremely fast reaction, being quantitative in 30 seconds. The

iodine solution is removed by an argon reverse flush and several acetonitrile washes, each followed by an argon reverse flush.

Other oxidizing agents such as sulfur[16] can be used in place of oxygen to create nucleotide phosphate analogs or to introduce radioactive atoms.

IMPORTANT: Do not stop the synthesis while the phosphorous is unoxidized.

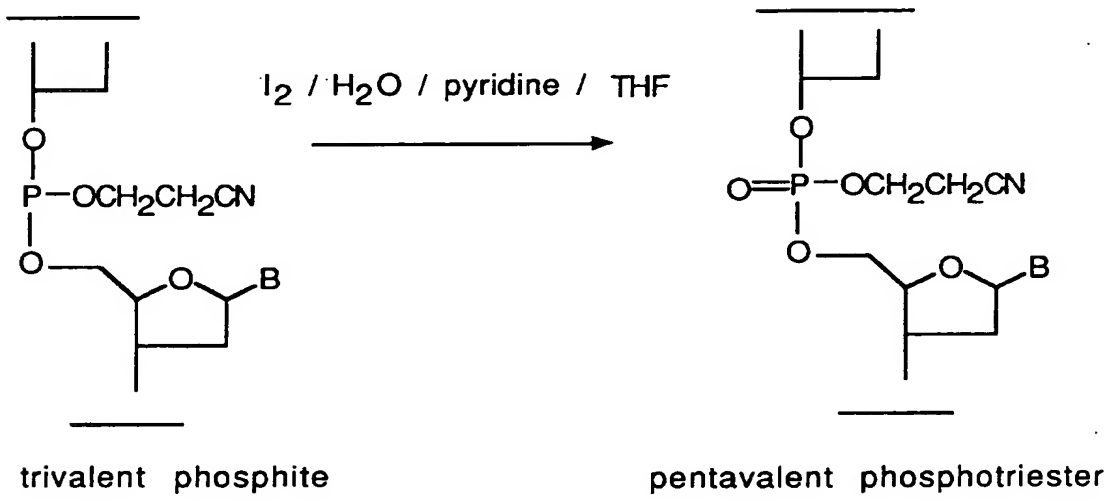


Figure 2-7
Oxidation of the trivalent phosphorous. The unstable trivalent phosphorous of the newly formed internucleotide linkage is oxidized to a stable pentavalent phosphorous using an iodine solution.

COMPLETION OF THE SYNTHESIS CYCLE

Following oxidation, a cycle of nucleotide addition is complete. The 5' terminus of the oligomer is protected by the dimethoxytrityl group. DNA synthesis continues by removing the 5' trityl and repeating another cycle of base addition. This is done until DNA of specified length has been fully synthesized.

Immediately after completing the synthesis, a trityl group may still be attached to the 5' terminus, according to the user's option. The ending method instructions may be programmed for the trityl group to remain attached (trityl on) or be cleaved (trityl off).

The oligonucleotides are usually detritylated when purifying by gel electrophoresis or ion exchange HPLC. The trityl groups are usually left intact when purifying by OPC or trityl-specific reverse phase HPLC. The ending method (trityl on or trityl off) is generally chosen before beginning a synthesis.

DEPROTECTION AND CLEAVAGE

When the synthesis is finished, the product and capped failure sequences (still attached to the support) exist as phosphate-protected, base-protected phosphotriesters. Complete deprotection is necessary to produce biologically active DNA. In addition, the oligonucleotides must be cleaved from the support. Cleavage and phosphate deprotection are performed automatically by the 380B. Base deprotection must be completed manually.

Cleavage:

Following synthesis, the DNA remains covalently attached to the support. The diester oligonucleotides are cleaved from the support by four consecutive 15-minute treatments with fresh, concentrated ammonium hydroxide. Alternatively, cleavage can be accomplished manually using the double syringe method[17]. As seen in Figure 2-8, the cleavage occurs at the base-labile ester linkage between the linker of the support and the 3' hydroxyl of the initial nucleoside. The cleaved DNA has a free 3' hydroxyl.

The column effluent from each treatment is forced into the DNA collection vial by an argon flush. The vial contains the crude mix (product and failure sequences) of base-protected oligonucleotides in ammonium hydroxide. Following cleavage, the column and the internal passages of the instrument are rinsed with acetonitrile and dried with argon. The used column and the DNA collection vial are removed. This leaves the synthesizer ready for another synthesis. The DNA is then base deprotected, desalted and purified for use.

Phosphate Deprotection:

The cyanoethyl protecting groups are also removed by treatment with ammonium hydroxide. This occurs at the same time as cleavage making phosphate deprotection very quick and simple (see Figure 2-8).

Base Deprotection:

The benzoyl and isobutyryl base protecting groups are removed by placing the vial of DNA at 55°C for 8 to 15 hours. This also cleaves the acetyl caps from the failure sequences. Base deprotection is an ammoniolysis reaction, where ammonia is a nucleophile, attacking the carbonyl of the amide protecting groups. For effective treatment, use fresh, concentrated ammonium hydroxide during cleavage. To ensure no decrease in ammonia concentration, store the reagent in a refrigerator, tightly capped. Discard thirty days after opening.

IMPORTANT: Use a tightly sealed DNA collection vial that can withstand positive pressure. The vial must also have a Teflon-lined cap. Rubber-lined caps have contaminants that leach out of the cap liner during deprotection. Teflon-lined caps can be ordered from Wheaton; Part Number 240408, size 13-425.

After completing deprotection, cool the ammonium hydroxide-DNA solution on ice to prevent losses from bubbling. Then remove the ammonia by vacuum. Ammonia is much easier to transfer at lower temperature than at room temperature. A note of caution: When drying down trityl-on syntheses, it is important to keep the oligonucleotide solution basic. Vacuum removal of the ammonia can lead to slightly acidic solution

conditions which may promote trityl removal. To maintain basic conditions during ammonia removal, add one drop of distilled triethylamine every 10 minutes. Also, avoid heating the sample.

If the 5' DMT group has been left on, it can be removed manually by treatment with 80% acetic acid/water for 20 minutes at room temperature. The acid is then diluted with ethanol and removed by vacuum, followed by several rinses with ethanol. This procedure is usually done after reverse phase HPLC or before radioactively labeling the 5' end prior to analysis by gel electrophoresis.

The deprotected, detritylated DNA has a free 5' and 3' hydroxyl and is biologically active. Desalting and purification may be necessary before use in experiments. An overview of these procedures is described later in this section. Details can be found in User Bulletin 13 (Revised).

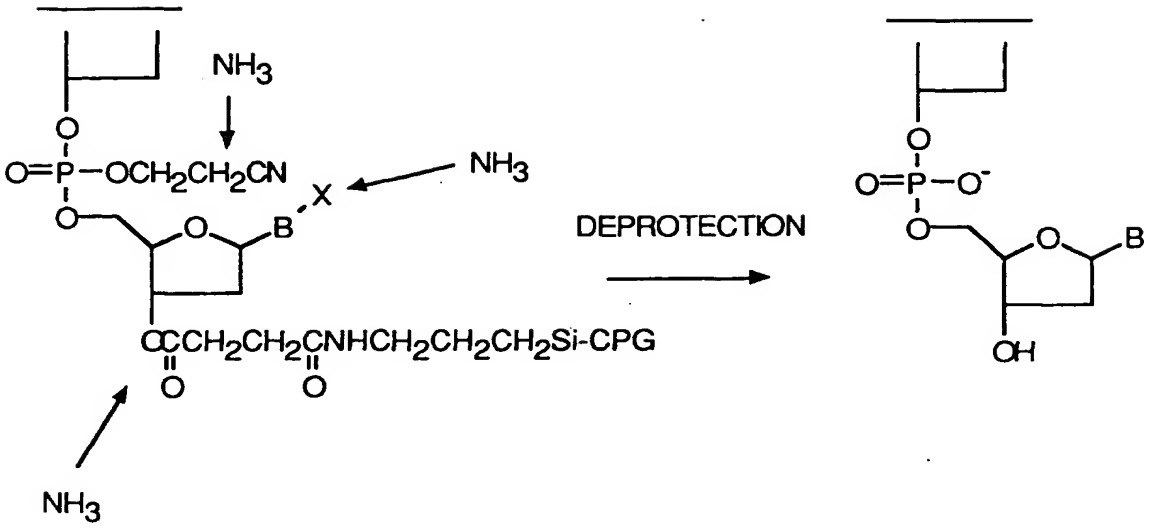


Figure 2-8
Deprotection and cleavage of ß-cyanoethyl protected oligonucleotides. Treatment with concentrated ammonium hydroxide removes the ß-cyanoethyl protecting groups and cleaves the oligonucleotides from the support. The benzoyl and isobutyryl base protecting groups (X) are removed by heating at 55°C in ammonia for 8 to 15 hours.

QUANTITATION OF THE OLIGONUCLEOTIDE

Nucleic acids of any variety are most easily quantitated by UV spectroscopy, measuring at or near their UV absorbance maxima, about 260 nm. A dilute aqueous solution of 1 ml or less, depending on the cuvette size, is measured by either scanning a region of about 200-350 nm or a single wavelength measurement. A scan of an oligonucleotide will show broad absorbance with a maxima near 260 nm. Using Beer's law, the concentration of the solution and absolute quantity can be calculated. As a useful approximation, 1 optical density unit (odu) of single-stranded oligonucleotide consists of about 33 micrograms, by mass. An approximation to relate absorbance to molar quantities is that a micromole of oligonucleotide has a number of odu equal to 10 times the number of bases. For example, a micromole of a 20mer would be 200 odu.

STORAGE OF THE OLIGONUCLEOTIDE

Most applications for synthetic oligonucleotides require less DNA than a synthesis provides. Fortunately, oligonucleotides can be stored easily, with little or no degradation for long periods of time. It is probably most convenient to store them refrigerated as a solution, in either a crude or purified state. The media may be concentrated ammonia, used to cleave and deprotect the crude oligonucleotide, water, or dilute buffer or salt. Typical aqueous media may contain ethanol, acetonitrile, triethylammonium acetate (TEAA), EDTA, etc. It is important to keep the oligonucleotides cold to minimize degradation and bacterial growth. Alternatively, oligonucleotides may be stored dried as a pellet in a clean, dry vessel, such as a microcentrifuge tube. The solution used to elute purified oligonucleotides from OPC, 20% acetonitrile, is a convenient and stable storage media. When stored by these means, oligonucleotides are stable for over a year. Avoid solutions that are mutagenic, oxidizing or outside the pH range of 3-12.

ANALYSIS AND PURIFICATION

This section reviews the most common methods for purification of synthetic oligonucleotides. HPLC (high performance liquid chromatography) and PAGE (polyacrylamide gel electrophoresis) can provide a high level of purity, but require initial capital investments and are labor intensive and time consuming. A short oligonucleotide (<30 bases) made with typical synthesis efficiency (>98% average DMT yield/cycle) may require a less stringent purification, with efficient desalting and removal of non-nucleoside synthesis byproducts. These methods are elaborated in detail in User Bulletin No. 13 Revised.

OPC

The Oligonucleotide Purification Cartridge (OPC, ABI part # 400771) was designed specifically for rapid, easy purification of synthetic oligonucleotides[18,19]. The method is based on a small, syringe-mounted cartridge containing an adsorbent material with an affinity for DMT oligonucleotides. The ammonia solution of the crude DMT oligonucleotide (10-20 crude odu) is applied directly to the cartridge. The DMT oligonucleotide product is retained. Byproducts, failure sequences not bearing a DMT group, and other impurities are not retained and are eluted. The DMT group of the OPC-bound oligonucleotide is removed with a mild acid solution, then the purified oligonucleotide is eluted (typically 1-5 odu) with about 1 ml of a 20% acetonitrile solution. The entire operation requires

15-20 minutes, and several OPC purifications can be conducted in parallel. Efficient, reliable purifications are achieved with oligonucleotides up to about 70 bases.

Several features of the OPC purification are noteworthy. No sample preparation is required. The OPC material is stable to concentrated ammonia. The ammonia solution provides a *denaturing* media, eliminating the purification complications of secondary structure and hydrogen-bonding. The DMT group is detached and retained in the cartridge. Thus, the purified, fully deprotected oligonucleotide is eluted in a small volume of 20% acetonitrile in water, completely desalted and ready for use.

PAGE

Polyacrylamide gel electrophoresis (PAGE) is a widely used method for the analysis and purification of oligonucleotides[21]. When a mixture of charged molecules are exposed to an electric field, they will migrate with velocities determined primarily by their mass to charge ratios. This ratio changes linearly with the log of the molecular weight of DNA of differing chain lengths. This allows a very ordered progression of oligonucleotides with mobility decreasing as the length increases. Proper PAGE technique can provide resolution, and preparative isolation, of single base length differences.

Oligonucleotide samples for PAGE are typically prepared by drying a known odu amount (by measuring absorbance) and redissolving in media such as formamide/1X TBE : 9/1 or 7M urea, which are denser

than the 1X TBE running buffer. These media and the gel matrix (containing 7M urea) should be denaturing to ensure disruption of secondary structure and hydrogen-bonding.

The most desirable format for gel electrophoresis of oligonucleotides is the slab gel. There are many commercial devices which essentially consist of a sandwich of two glass plates held apart by two side spacers. The thickness of the spacers determine the gel thickness. For UV shadowing, .75 - 1.5 mm is convenient. The acrylamide solution is poured in between and allowed to polymerize. A comb is also inserted, which forms wells where the olignuclotide is loaded. Each well forms a vertical lane during the electrophoresis. The plate length should be about 16-40 cm, depending on oligonucleotide length. The width depends only on the number of samples to be run. Dyes, such as bromophenol blue and xylene cyanol are run either in the samples or by themselves as indicators of the migration distance of the oligonucleotides. The acrylamide concentration of the gel matrix determines the velocity of the oligonucleotides. A range of 8-20% is typical. Longer oligonucleotides require lower acrylamide concentration.

When the dyes indicate the appropriate migration distance of the oligonucleotide product, the power is turned off and the gel sandwich is disassembled. There are several methods of analysis: UV shadowing, staining, and radiolabelling/autoradiography.

UV shadowing:

This is the simplest, easiest, and introduces the fewest artifacts into the analysis. Depending on oligonucleotide length, from 0.5 to 10 odu are loaded in a well. After electrophoresis, the gel is transferred from the plates to a clear plastic wrapped, fluorescent TLC plate, most conveniently plastic-backed, 20x20cm. The gel is visualized with a UV lamp at short wavelength (254 nm). The bands appear dark against a fluorescent green background. A permanent record can be made by photography through a green filter.

Staining:

Dyes such as methylene blue, ethidium bromide, Stains-All, and others, will visualize oligonucleotides in a polyacrylamide matrix. After electrophoresis, the gel is transferred to a pan containing the staining agent and let soak for some time period. This technique is more sensitive than UV shadowing, but is more time-consuming and uncertain than UV shadowing.

Radiolabelling:

This is the most sensitive, but most laborious method of analysis of oligonucleotides. Typically about 0.01 odu is phosphorylated enzymatically with T4 polynucleotide kinase and gamma-^{32}P ATP to give 5' ^{32}P phosphorylated oligonucleotides. Alternatively, ^{35}S ATP can be used, and the oligonucleotides can be labelled at either the 5' or 3' terminus. The radiolabelled samples are electrophoresed on the gel. The gel is wrapped in plastic and exposed to X-ray film in a dark room. An autoradiogram is generated in a time period ranging from minutes to days, depending on the specific activity of the ATP and other

radiolabelling parameters. The film has a finite capacity for development and exposure time must be carefully monitored, usually by taking several exposures. The gel pattern on the film may be quantitated by densitometry. Product identification is obvious when the appropriate tracking dye or oligonucleotide standard is also present on the gel.

By any of these techniques, the gel pattern of electrophoresis of an oligonucleotide can be very diagnostic about the course of synthesis. Many synthesizer and reagent problems can be diagnosed by the appearance and relative amounts of the "failure bands". Also, PAGE has a distinct advantage in that many samples can be analyzed and purified concurrently. The equipment is relatively inexpensive and easy to maintain. For bigmers, PAGE is usually the most efficient purification method and the only analytical method. The primary disadvantages of PAGE are that it is labor intensive and dependent on good technique.

Oligonucleotides can be purified by locating the product band by UV shadowing and excising the gel material therein with a clean razor blade. The gel material which is removed should be free of failure bands, most typically the lower N-1 band. In a preparative electrophoresis run, the product is run further, on a thicker gel, than in an analytical run, to maximize the separation of the product band from the N-1 band. The excised gel fragment(s) is soaked in an elution buffer. The gel debris is then removed by a desalting method, such as with an OPC cartridge.

HPLC

High Performance Liquid Chromatography (HPLC) is another efficient method which combines quantitative analysis and purification of oligonucleotides. One of the advantages of HPLC is a high level of automation. Systems are available which allow for repetitive programmed injection, analysis, and data manipulation and storage. Two different types of column adsorbents are popular for oligonucleotides; reverse-phase and ion-exchange.

Reverse-phase adsorbents discriminate by the hydrophobic differences between oligonucleotides of varying lengths and sequences. When the 5' DMT is on the oligonucleotide, this group is dominant in its interaction with the support adsorbent. Reverse-phase columns also can adequately resolve DMT off oligonucleotides with sufficient capacity. The mobile phase is typically a volatile buffer such as 0.1M triethylammonium acetate. The oligonucleotides are eluted with a gradient of increasing organic solvent, such as acetonitrile. These conditions are non-denaturing. Occasionally, certain sequences can exhibit unpredictable HPLC elution patterns, caused by inter- or intramolecular secondary structure and hydrogen-bonding effects.

Ion-exchange adsorbents elute oligonucleotides based on increasing charge, i.e. chain length. An increasing salt gradient in the mobile phase is used to displace the oligonucleotide phosphate anions. The salt anions, such as ammonium sulfate or sodium phosphate, and the DNA pair with the adsorbent-bound cation, usually alkylated ammonium species. Since ion-exchange analysis separates only on the basis of

increasing charge, the desired product oligonucleotide will always elute after the lesser charged failure sequences. The high salt mobile phase also provides a denaturing media. Both of these factors allow easy product identification. Preparative ion-exchange HPLC requires a final desalting operation, most efficiently conducted with OPC.

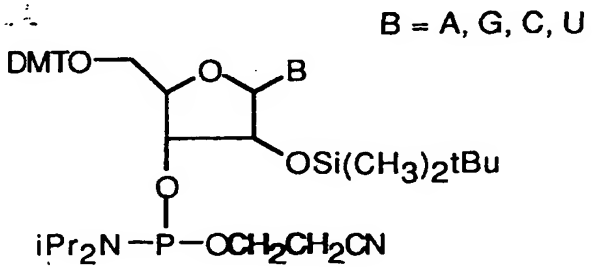
For more information, consult the chapter on HPLC in User Bulletin 13, Revised.

ALTERNATE CHEMISTRIES

In addition to the phosphoramidite chemistry method to prepare "normal" phosphodiester oligonucleotides, many alternative chemistries have been demonstrated on ABI synthesizers. These other products include; synthetic RNA, phosphate-analog oligonucleotides, and chemically derivatized oligonucleotides which can be covalently attached to other molecules.

RNA Synthesis

RNA oligoribonucleotides can be synthesized on the Model 380B[22,23]. Using 2' silyl 5' DMT cyanoethyl phosphoramidite RNA monomers, the only cycle change is to increase the coupling wait time of the corresponding DNA synthesis cycle to 600 seconds. RNA monomers bear a large 2' silyl protecting group and therefore require longer time to couple. Details can be found in Applied Biosystems User Bulletins No. 47 and 53.



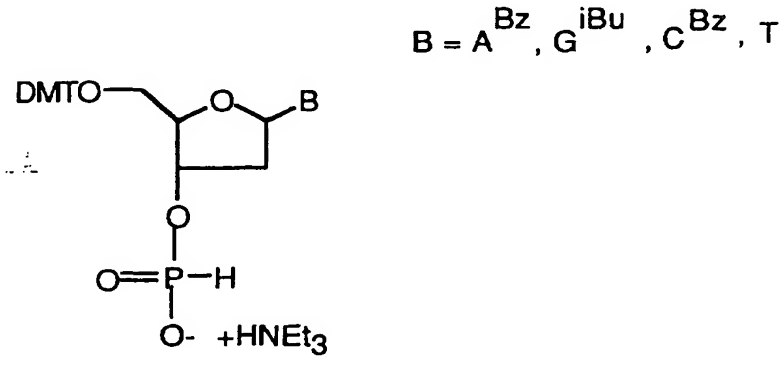
RNA phosphoramidites

Figure 2-9

Hydrogen-Phosphonate Chemistry

Hydrogen-phosphonate chemistry is useful and effective for preparing either "normal" phosphodiester oligonucleotides or phosphate-analog oligonucleotides[24]. Although the cycle is similar in that it contains coupling, capping[25], and detritylation steps, the reagents[26] are different from those used for the phosphoramidite method. User Bulletin No. 44 provides details regarding appropriate synthesis reagents and post-synthesis protocols.

The synthesis efficiency of hydrogen-phosphonate chemistry is routinely less than that of the phosphoramidite method (typically 95-96% trityl yields). The primary advantage of hydrogen-phosphonate chemistry is the potential for one-step, post-synthesis conversion of the internucleoside hydrogen-phosphonate groups to a variety of phosphate species[27] such as phosphodiesters, phosphoramidates, phosphotriesters and phosphorothioates.

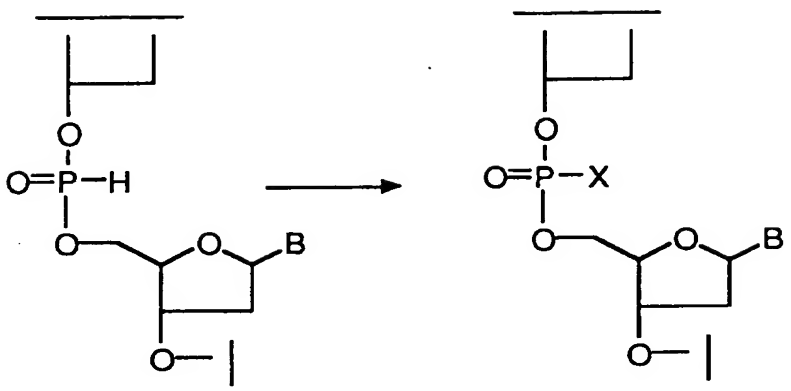


hydrogen-phosphonate monomers

Figure 2-10

Phosphorothioate DNA

Once hydrogen-phosphonate synthesis of an oligonucleotide is completed, the column may be removed and a sulfurizing reagent introduced by the double-syringe method[17,28]. All the internucleoside hydrogen-phosphonates are rapidly converted to phosphorothioates. After regular ammonia cleavage/deprotection, oligonucleotides bearing phosphorothioate linkages behave chemically similarly to "normal" phosphodiester DNA. They show the same electrophoresis and HPLC behavior. This class of phosphate-analog DNA has shown activity in anti-sense translation arrest experiments. In particular, both a sequence, and non-sequence specific effect has been observed in inhibiting in vitro viral replication of HIV[29]



CONVERSION OF HYDROGEN-PHOSPHONATE
TO PHOSPHATE ANALOGS

X	OXIDANT
O	I_2/pyr/Et_3N/H_2O/THF
S	sulfur/CS_2/pyr/Et_3N
NR_2	HNR_2/CCl_4
OR	ROH/base/CCl_4

Figure 2-11

5' Attachments

In the last several years many applications have been identified and developed for the covalent attachment of small molecules to oligonucleotides. These molecules include fluorescent dyes[30], biotin[31], proteins[32], and other species that allow the identification of oligonucleotides in biological systems. In addition, oligonucleotides can be derivatized to allow attachment to solid supports[33]. In this way, for example, an affinity matrix can be constructed to purify a sequence complementary to the support-bound sequence.

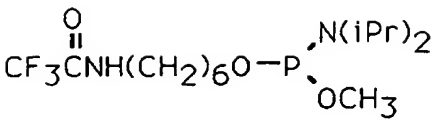
The attachment can be made at several sites on the oligonucleotide. However, this site must not impair hybridization or confer chemical instability. For these reasons and for synthesis ease and efficiency, the 5' terminus is usually the preferred location for derivatization of an oligonucleotide.

Fluorescent-dye Linked Sequencing Primers

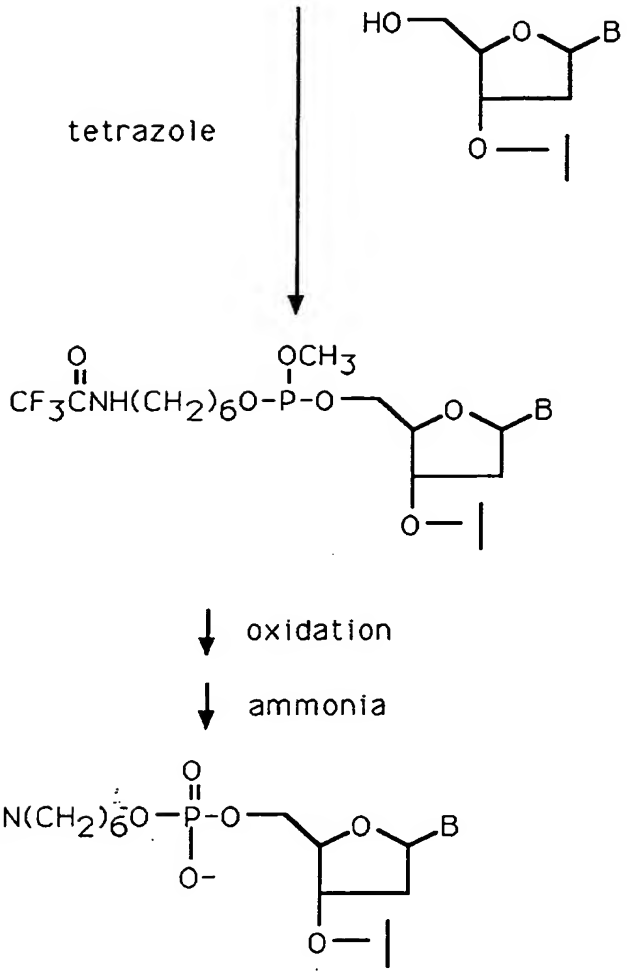
Aminolink 2:

The 5' fluorescent dye labelled sequencing primers used for the Applied Biosystems 370A DNA Sequencer consist of three parts; the oligonucleotide primer, a linker, and a fluorescent dye. The linker bears a highly nucleophilic primary amine group which reacts with the electrophilic N-hydroxy succinimide group of the fluorescent dye. This linker group is created with AMINOLINK 2 (ABI Part No. 400808)[34]. Aminolink 2 is a phosphoramidite molecule with a six carbon chain and a protected amine group (Figure

2-12). Although it comes as a viscous liquid rather than as a powder, this reagent is handled and used like a phosphoramidite nucleoside. Activation with tetrazole forms an active intermediate that couples to the 5' hydroxyl terminus of the support-bound oligonucleotide, in the final coupling cycle. Oxidation and ammonia cleavage/deprotection yields the aminolink-oligonucleotide, in solution. Coupling to the fluorescent dye-NHS ester, or other electrophilic species, is conducted in a homogeneous solution.



Aminolink 2



aminolinked oligonucleotide (in solution)

Figure 2-12

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17. The DOUBLE-SYRINGE METHOD for manual ammonia cleavage:

1. Attach an empty luer tip syringe, with plunger fully inserted, into one end of the synthesis column.
2. Load 2 ml of conc. ammonia in another luer tip syringe and attach to the other end of the column
3. Holding a syringe in each hand, carefully inject the reagent through the column to the empty syringe and return the reagent through the column several times.
4. Allow it to stand for at least one hour at room temperature.
5. Drain all of the reagent into one syringe, detach and eject contents carefully into an appropriate vial for heating to achieve complete deprotection.

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